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Sequence breakpoints in the aflatoxin biosynthesis gene cluster and flanking regions in nonaflatoxigenic *Aspergillus flavus* isolates

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Abstract

Aspergillus flavus populations are genetically diverse. Isolates that produce either, neither, or both aflatoxins and cyclopiazonic acid (CPA) are present in the field. We investigated defects in the aflatoxin gene cluster in 38 nonaflatoxigenic *A. flavus* isolates collected from southern United States. PCR assays using aflatoxin-gene-specific primers grouped these isolates into eight (A–H) deletion patterns. Patterns C, E, G, and H, which contain ⩾40 kb deletions, were examined for their sequence breakpoints. Pattern C has one breakpoint in the *cypA* 3′ untranslated region (UTR) and another in the *verA* coding region. Pattern E has a breakpoint in the *amdA* coding region and another in the *ver1* 5′UTR. Pattern G contains a deletion identical to the one found in pattern C and has another deletion that extends from the *cypA* coding region to one end of the chromosome as suggested by the presence of telomeric sequence repeats, CCCTAATGTTGA. Pattern H has a deletion of the entire aflatoxin gene cluster from the *hexA* coding region in the sugar utilization gene cluster to the telomeric region. Thus, deletions in the aflatoxin gene cluster among *A. flavus* isolates are not rare, and the patterns appear to be diverse. Genetic drift may be a driving force that is responsible for the loss of the entire aflatoxin gene cluster in nonaflatoxigenic *A. flavus* isolates when aflatoxins have lost their adaptive value in nature.
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1. Introduction

Aflatoxins are toxic and hepatocarcinogenic polyketides produced by several species in *Aspergillus* section *Flavi* (Payne and Brown, 1998). *Aspergillus flavus*, the most common causal fungus, produces aflatoxins B_1 and B_2. Its close relative, *Aspergillus parasiticus*, produces aflatoxins B_1, B_2, G_1, and G_2. Enzymes and regulatory proteins for aflatoxin synthesis in *A. flavus* and *A. parasiticus* are encoded by more than two dozen clustered genes in a 66 kb region (Ehrlich et al., 2005; Yu et al., 2004). *A. flavus* also produces an indole-tetramic acid toxin called cyclopiazonic acid (CPA) whereas *A. parasiticus* does not produce CPA (Blaney et al., 1989; Dorner et al., 1984; Takahashi et al., 2004). CPA is a highly selective inhibitor of calcium-dependent ATPase in skeletal muscle (Burdyga and Wray, 1999) that can cause hepatic and splenic necrosis, and inflammation in chicken (Dorner et al., 1983), but its significance to human health has not yet been fully investigated. Synthesis of fungal toxins by gene clusters is well established (Cary et al., 2001). A gene cluster may be responsible for CPA production, but no genetic studies on CPA formation have been carried out. CPA and aflatoxins have been found in corn and peanuts as co-contaminants (Fernandez Pinto et al., 2001; Lee and Hagler, 1991; Urano et al., 1992).

The occurrence of aflatoxins in food and feed commodities continues to be a potential threat to consumer

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safety and to the world markets for US export due to the extremely low tolerance levels (Cleveland et al., 2003). To date, no regulatory limits have been set for CPA because its natural occurrence in human food appears to be low and its occurrence in agricultural products susceptible to aflatoxin contamination is indirectly controlled by regulations in place for aflatoxins. To minimize potential human exposure to aflatoxins, biological control strategies of using nonaflatoxigenic *A. flavus* isolates to competitively exclude toxigenic *A. flavus* strains are being tested to reduce aflatoxin contamination of susceptible crops in the field (Antilla and Cotty, 2002; Dorner, 2004a,b). Two *A. flavus* isolates, AF36 and NRRL 21882, were recently registered as biopesticides with the United States Environmental Protection Agency for the management of aflatoxin-producing fungi. AF36 has been applied to over 30,000 acres in Arizona and Texas since 1999 to decrease aflatoxin contamination of cottonseed (Antilla and Cotty, 2002). NRRL 21882 reduced aflatoxin levels in peanuts by 70–90% in field tests and is the active ingredient of the commercial biopesticide Afla-Guard (Dorner, 2004a,b).

Populations of *A. flavus* in many parts of the world vary considerably in the proportion of isolates that are aflatoxigenic (Pildain et al., 2004; Takahashi et al., 2004; Tran-Dinh et al., 1999; Vaamonde et al., 2003). Horn and Dorner (1999) examined the aflatoxigenicity of *A. flavus* soil isolates along a transect extending from New Mexico to Virginia and reported regional differences in production of aflatoxins, ranging from >95% of isolates in the peanut-growing regions of southern Alabama and Georgia to approximately 50% in eastern North Carolina and Virginia. Although *A. flavus* and *A. parasiticus* both contain the aflatoxin gene cluster with genes in the same sequential order (Ehrlich et al., 2005; Yu et al., 2004), *A. parasiticus* isolates that do not produce aflatoxins are rare (Horn et al., 1996; McAlpin et al., 1998; Tran-Dinh et al., 1999).

Bilgrami et al. (1988) have postulated that aflatoxin-producing ability and associated morphological traits are maintained in nature by competition with other microorganisms and by growth under suboptimal conditions. The molecular mechanisms responsible for the loss of aflatoxin production in *A. flavus* isolates are not well understood, although a history of recombination may account for some of the variability in aflatoxigenicity in *A. flavus* populations (Geiser et al., 1998). A defect in the aflatoxin pathway gene, *pksA*, in AF36 has been identified and found to be associated with the loss of aflatoxin production (Ehrlich and Cotty, 2004). The loss of aflatoxin-producing ability in *A. flavus* 649-1 has been suggested to be associated with a large deletion in the aflatoxin gene cluster (Prieto et al., 1996). *Aspergillus oryzae* is closely related to *A. flavus* but does not produce aflatoxins (Kurtzman et al., 1986). This domesticated species has been widely used in industrial enzyme production and food fermentation. Kusumoto et al. (2000) have reported deletions in the aflatoxin gene cluster in some *A. oryzae* isolates. On the basis of these deletions, they categorized *A. oryzae* isolates into three groups, 1, 2, and 3.

The present work is part of a study investigating genetic variability of *A. flavus* populations in the United States. We examined and characterized deletions in the aflatoxin gene cluster in nonaflatoxigenic *A. flavus* isolates that either produce or do not produce CPA (Horn and Dorner, 1998, 1999). We also determined sequence breakpoints associated with various deletions in the aflatoxin gene cluster of *A. flavus* isolates.

2. Materials and methods

2.1. Fungal isolates

Aspergillus flavus isolates used in this study are listed in Table 1. Isolates were collected from agricultural soils of southern United States (Horn and Dorner, 1998, 1999), except for NRRL 21882 (=NPL 45), which is the active ingredient of the Afla-Guard biopesticide and was obtained from a Georgia peanut seed in 1991 (Dorner, 2004). Isolates previously were examined for aflatoxin and CPA production, and were categorized into vegetative compatibility groups (VCGs) (Horn and Dorner, 1999). Of the 38 nonaflatoxigenic isolates, 15 are CPA-positive and 23 are CPA-negative. All isolates belong to the L-strain morphotype of *A. flavus*, which produces sclerotia that are >400 μm in diameter (Cotty, 1989; Horn and Dorner, 1999). Isolate GA13-9, which produces aflatoxins and CPA, was used as a positive control in PCR assays of genes in the aflatoxin gene cluster and flanking regions.

2.2. Culture conditions and isolation of genomic DNA

Adye and Mateles (1964) medium was used to grow submerged cultures in a gyroshaker for preparation of genomic DNA. DNA was prepared using a GenElute Plant Genomic DNA Miniprep kit (Sigma, St. Louis, Missouri, USA). Potato dextrose agar (PDA) (Difco, Detroit, Michigan, USA) plates were used for the examination of morphology of *A. flavus* isolates. The PDA plates were incubated at 30 °C in darkness for 7 days.

2.3. PCR primers

Primers were derived from aflatoxin biosynthetic pathway genes of *A. flavus* when they were available in the GenBank database. Otherwise, primers were derived from aflatoxin genes of *A. parasiticus* NRRL 5862 (=SU-1) (AY371490) or closely-related *A. oryzae* RIB40. The *norB- cypA* primer set used to amplify unique deletion regions found in *A. flavus* was based on Ehrlich et al. (2004). Primers for the upstream 9 kb region

Table 1
A. flavus strains used in the study

Isolate[a]	Geographic origin	Source	Characteristics[b]	Deletion[c]	Sequence[d]
NPL LA4-5	Natchitoches Pa.	Corn field[e]	AF^{-}, CPA^{+}, C^{++}	A	—
MS5-6	Covington Co.	Peanut field	AF^{-}, CPA^{+}, C^{++}	A	—
NM1-6	Lea Co.	Corn field	AF^{-}, CPA^{+}, C^{++}	A	—
SC6-9	Calhoun Co.	Cotton field	AF^{-}, CPA^{+}, C^{++}	A	—
TX9-8	Jones Co.	Cotton field	AF^{-}, CPA^{+}, C^{++}	A	—
VA2-1	King & Queen Co.	Corn field	AF^{-}, CPA^{+}, C^{++}	A	—
MS1-1	Franklin Co.	Corn field	AF^{-}, CPA^{+}, C^{++}	B	—
NC3-6	Edgecombe Co.	Peanut field	AF^{-}, CPA^{+}, C^{++}	B	—
SC3-5	Lee Co.	Peanut field	AF^{-}, CPA^{+}, C^{++}	B	—
TX21-9	Navarro Co.	Cotton field	AF^{-}, CPA^{+}, C^{++}	B	—
AL3-9	Conecuh Co.	Peanut field	AF^{-}, CPA^{+}, C/S	C	AY864289
NC5-2	Duplin Co.	Corn field	AF^{-}, CPA^{+}, C/S	C	AY864290
VA4-4	Surry Co.	Peanut field	AF^{-}, CPA^{+}, C/S	C	AY864291
GA4-4	Laurens Co.	Soybean field	AF^{-}, CPA^{+}, C^{++}	D	—
GA13-9	Terrell Co.	Peanut field	AF^{+}, CPA^{+}, C^{++}	D	—
LA10-4	Concordia Pa.	Cotton field	AF^{-}, CPA^{+}, C^{++}	D	—
TX13-5	Comanche Co.	Peanut field	AF^{-}, CPA^{-}, C^{++}, 76	E	AY987856
TX21-5	Navarro Co.	Cotton field	AF^{-}, CPA^{-}, C^{++}, 71	E	AY987855
NC7-8	Bladen Co.	Peanut field	AF^{-}, CPA^{-}, C^{++}, 79	F	—
AL1-4	Clarke Co.	Soybean field	AF^{-}, CPA^{-}, S^{++}, 73	G	AY995194
TX20-9	Navarro Co.	Cotton field	AF^{-}, CPA^{-}, S^{++}, 75	G	AY995193
AL4-3	Conecuh Co.	Peanut field	AF^{-}, CPA^{-}, S^{++}, 70	H	AY966015
AL4-7	Conecuh Co.	Peanut field	AF^{-}, CPA^{-}, S^{++}, 64	H	AY970954
GA1-3	Screven Co.	Peanut field	AF^{-}, CPA^{-}, S^{++}, 67	H	AY970955
GA2-9	Bulloch Co.	Peanut field	AF^{-}, CPA^{-}, S^{++}, 69	H	AY970956
GA3-3	Emanuel Co.	Cotton field	AF^{-}, CPA^{-}, S^{++}, 64	H	AY966016
MS1-7	Franklin Co.	Corn field	AF^{-}, CPA^{-}, S^{++}, 67	H	AY970957
MS2-2	Lincoln Co.	Corn field	AF^{-}, CPA^{-}, S^{++}, 69	H	AY970958
MS6-8	Covington Co.	Peanut field	AF^{-}, CPA^{-}, S^{++}, 64	H	AY970959
NC6-1	Sampson Co.	Soybean field	AF^{-}, CPA^{-}, C/S, 64	H	AY970960
NC6-7	Sampson Co.	Soybean field	AF^{-}, CPA^{-}, S^{++}, 69	H	AY970961
NC7-1	Bladen Co.	Peanut field	AF^{-}, CPA^{-}, S^{++}, 74	H	AY970962
SC5-1	Sumter Co.	Peanut field	AF^{-}, CPA^{-}, S^{++}, 67	H	AY970963
SC5-6	Sumter Co.	Peanut field	AF^{-}, CPA^{-}, S^{++}, 78	H	AY970964
SC6-10	Calhoun Co.	Cotton field	AF^{-}, CPA^{-}, S^{++}, 64	H	AY970967
TX9-2	Jones Co.	Cotton field	AF^{-}, CPA^{-}, S^{++}, 70	H	AY970965
TX16-3	Hill Co.	Peanut field	AF^{-}, CPA^{-}, S^{++}, 67	H	AY970966
VA1-6	Essex Co.	Corn field	AF^{-}, CPA^{-}, S^{++}, 64	H	AY970953
NRRL 21882	Terrell Co.	Peanut seed	AF^{-}, CPA^{-}, S^{++}, 24	H	AY966014

[a] NPL, National Peanut Research Laboratory Culture Collection, Dawson, Georgia; NRRL, Agricultural Research Service Culture Collection, Peoria, Illinois. The capital letter abbreviation for NPL designations indicates the state from which an isolate was collected.

[b] AF, aflatoxin; CPA, cyclopiazonic acid. C^{++}, heavily conidial on PDA; S^{++}, predominantly sclerotial on PDA. C/S, intermediate levels of conidia and sclerotia produced on PDA. The number indicates the VCG to which an *A. flavus* isolate belongs (Horn and Dorner, 1999). All are L-strain isolates, which produce sclerotia >400 μm in diameter.

[c] Deletion patterns in the aflatoxin biosynthesis gene cluster (see Fig. 1).

[d] GenBank accession numbers of sequences containing sequence breakpoints.

[e] All NPL field isolates collected from soil.

beyond the *norB* gene end of the aflatoxin gene cluster and the downstream sugar utilization gene cluster (see Fig. 1) were derived from *A. flavus* AF13 (AY510451) and *A. parasiticus* NRRL 5862, respectively. The sequences of paired primers, which amplified PCR products in the range of 0.5–1.0 kb, are summarized in Table 2.

2.4. PCR conditions

PCR screenings for deletions in the aflatoxin gene cluster and its flanking regions were performed in a Perkin–Elmer GeneAmp PCR System 2400. Fifty microliter of each PCR mixture was heated at 94 °C for 5 min and then subjected to 30 cycles consisting of denaturation at 94 °C for 30 s, annealation at 55 °C for 30 s, and extension at 72 °C for 1.5 min. A final 7-min extension step at 72 °C was included. A 5 μl aliquot of each PCR product was examined on 1.0% agarose gel (Bio Rad, Richmond, California, USA) in Tris–borate–EDTA buffer, stained with ethidium bromide (0.5 mg/ml) for 10 min, and visualized under UV light. For the Inverse PCR protocol used to amplify regions flanking the sequence breakpoint junction (see below), a PCR amplification of 35 cycles was performed instead.

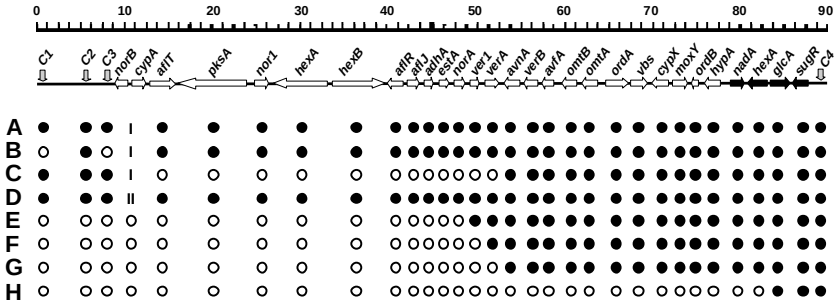

Fig. 1. Deletion patterns in the aflatoxin gene cluster of *A. flavus* isolates. Solid circles indicate positive PCR products and open circles indicate no PCR products. (I) Type I deletion in the *norB-cypA* region which yields a PCR product of 0.3 kb. (II) Type II deletion in the *norB-cypA* region which yields a PCR product of 0.8 kb. The region containing *nadA-hexA-glcA-sugR* represents the sugar utilization cluster. C1, C2, C3, and C4 are in the flanking regions of the aflatoxin gene cluster and the sugar utilization gene cluster.

Table 2
PCR primer sets derived from aflatoxin biosynthetic genes and flanking regions

Designation	Forward primer	Reverse primer
C1	CGTTCCAGTAGTTCGTATCG	CATCGTAAACGTTGACACAG
C2	TCGCCTTGTTCTCGCTATAC	ACACCTGATAGCGAGAGTTC
C3	GCGATCTGTAACACTACACA	GCCATACGATTCCCAAGTCT
norB-cypA	GTGCCCAGCATCTTGGTCCA	AGGACTTGATGATTCCTCGTC
aflT	ATGACATGCTAATCGACGAG	AGGCGCATGCTACGGATC
pksA(pksL)	ACTTTGAGGGCGTTCTGTGC	CTTTCGGTGGTCGGTGATTC
nor1	AGCACGATCAAGAGAGGCTC	GATCTCAACTCCCCTGGTAG
fasA(hexA)	TCCTATCCAGTCCACCTCGTA	CACATCTTTGTCTTGCCCGC
fasB(hexB)	ACAATCGAATGACAACACTGC	CCACCGAATCCACTACCTACA
aflR	ATGGTCGTCCTTATCGTTCTC	CCATGACAAAGACGGATCC
aflJ	CTTCAACAACGACCCAAGGTT	AGATGAGATACACTGCCGCA
adhA	CCTCGTGGGAGAGCCAAATC	GGAGCAAGAAGGTTACAGCG
estA	CGATGGGACTGACGGTGATT	ACCACGCCGCTGACTTTAT
norA	GTGTTCGTGTGTCGCCCTTA	GTCGGTGCTTCTCATCCTGA
ver1	CATCGGTGCTGCCATCGC	CCTCGTCTACCTGCTCATCG
verA	CCGCAACACCACAAGTAGCA	AAACGCTCTCCAGGCACCTT
avnA	GCGATAGAACTGACAAAGGCA	GAATGAGTCTCCAAAGGCGAG
verB	TTCAGTGACAAAGGTCTTCGC	GGCAGCGTT ATTGAGCATCT
avfA	ATTCAAATCCTCGTTCGGTCG	TAGCCCGTTGGTTGTGTTCC
omtB	ACAGACGATGTGGGCAAACG	ACGCAGTCCTTGTTAGAGGTG
omtA	CAGGATATCATTGTGGACGG	CTCCTCTACCAGTGGCTTCG
ordA	AAGGCAGCGGAATACAAGCG	ACAAGGGCGTCAATAAAGGGT
vbs	AACGAGCAGCGTAAGGGTCT	TCAGCCAGAGCATACACAGTG
cypX	GGAGCCTACCATTCGCAACA	GGCTTTGACGAACAGATTCCG
moxY	TGCTACTGGAACGAAGACCG	CGACGACAACCAAACGCAA
ordB	GCTGCTACTGGAATGAAGACC	ATGCGACGACAACCAAACG
hypA	CGCAAGACGGCAGAGATACT	GCTCCTTCAGTTCCACACCA
nadA	TGACGAGGCCTGCGAGCTGT	AAGCCTCTTCAGAACGGTCA
hexA	TGTCCTCACCTCTGGCGTAT	AGACCAACCACTCTTATGGGC
glcA	AGACACAGTCATCGCCTGTT	GGTGCGAATAGGTGCAGGTA
sugR	TCAGCTGAAGCGCTCGAGAG	GTATTGCCGCACTATGTATG
C4	ATCGTGCAGACAGGAACAC	GGTGCCTTGGCCTATGCGCT

2.5. Determination of sequence breakpoints in or beyond the aflatoxin gene cluster by inverse PCR

An Inverse PCR protocol (Ochman et al., 1988) was used to obtain the DNA fragments flanking the breakpoints of isolates with pattern E (TX13-5 and TX21-5), pattern G (AL1-4 and TX20-9), and pattern H (GA1-3, NC7-1, SC5-6, and NRRL 21882). Before carrying out Inverse PCR, we performed high-resolution PCR analyses using several sets of primers to locate the smallest region that putatively contained breakpoints. Restriction sites in and around the smallest region were then mapped for subsequent selection of suitable restriction enzymes for genomic DNA digestion. Approximately 0.2 μg genomic DNA of each isolate was digested to completion with 10 U of restriction enzymes at 37 °C for 4 h. For TX13-5 and TX21-5, genomic DNA was digested with *Bam*HI, *Eco*RV, *Nde*I, *Sma*I, and *Stu*I. For AL1-4 and TX20-9, genomic DNA was digested with *Bam*HI, *Eco*RV, *Kpn*I, *Sma*I, and *Sph*I. For GA1-3, NC7-1, SC5-6, and NRRL

21882, genomic DNA was digested with *Bgl*II and *Stu*I. The digested genomic DNA was purified by a DNA Clean & Concentrator-5 kit (ZYMO RESEARCH, Orange, California, USA). The purified digested mix was ligated with 3 U of ligase at 37 °C for 2 h, purified with the DNA Clean & Concentrator-5 kit, and eluted with deionized water in a final volume of 6 μL. Two primers (V55: catcggtgctgccatcgcc and 1160Comp: tacgcagctatggatggttgg for TX13-5 and TX21-5; verA1291R: aaacgctctccaggcacctt and IP98avnA: ggaaaggaacgca acaagactt for AL1-4 and TX20-9; glcF: agacacagtcatcgcctgtt and glcD: atggtatagacaaccgtcgat for GA1-3, NC7-1, SC5-6, and NRRL 21882) were used in the first round of PCR in which 3 μL of the digested and ligated mix was added to 50 μL Plantinum Blue PCR Supermix (Invitrogen, Carlsbad, California, USA). A nested primer (Comp1020: gctgagaataggagcaggctc) located approximately 180 nt upstream of the Comp1160 was used for TX13-5 and TX21-5, and another nested primer (glcU: cacactgcaatgttctctcc) approximately 180 nt upstream of the gluD primer was used for GA1-3, NC7-1, SC5-6, and NRRL 21882 in a second round of PCR with the purified first PCR product as the template to confirm the authenticity of the first PCR product. PCR products were sequenced at Iowa State University DNA Sequencing and Synthesis Facility (Ames, Iowa, USA).

2.6. Determination by conventional PCR of sequence breakpoints responsible for deletion of the entire aflatoxin gene cluster

The conventional PCR technique was used to determine the breakpoints in the other 14 *A. flavus* isolates from pattern H (Table 1). A flanking region at one end of the complete deletion of the aflatoxin gene cluster of GA1-3, NC7-1, SC5-6, and NRRL 21882 contained telomeric repeats (see Sections 3 and 4); therefore, a telomere-derived primer, TeloR: atgttgaccctaatgttgacccta and the glcU primer were used in PCR. A primer ratio of glcU to TeloR equal to 4:1 was used. The PCR products were gel-purified with a Zymoclean Gel DNA Recovery kit (ZYMO RESEARCH) and sequenced with glcU or P-BK: tctgaatttcggcctcatgc, which is approximately 80 nt downstream of the breakpoint junction of GA1-3.

2.7. Single nucleotide polymorphisms in the omtA gene of A. flavus isolates

A portion of the *omtA* gene in *A. flavus* isolates was PCR amplified by the *omtA* primers (Table 2) and sequenced. This 594 bp genomic region contains introns I, II, and III, and coding regions for amino acids 97–237. Multiple sequence comparison was performed by using the DNAMAN software (Lynnon BioSoft, Quebec, Canada) to locate single nucleotide polymorphisms (SNPs) in the *omtA* sequences.

3. Results

3.1. Deletions in the aflatoxin gene cluster of nonaflatoxigenic A. flavus isolates

Using PCR with aflatoxin-gene-specific primers (Table 2), we grouped the aflatoxin-positive control isolate (GA13-9), 15 aflatoxin-negative/CPA-positive isolates, and 23 aflatoxin-negative/CPA-negative isolates into eight deletion patterns (Fig. 1). In this study, the region beyond the *norB* gene is referred to as the "left" side of the aflatoxin gene cluster, and the region beyond the *hypA* gene is referred to as the "right" side of the aflatoxin gene cluster. Compared to the *norB-cypA* region of *A. parasiticus* (AY371490), two types of deletions were found in *A. flavus* isolates. Type 1 in patterns A–C results from the deletion of the coding regions for amino acid residues 1–280 of NorB and 1–112 of CypA, and the *norB-cypA* intergenic region. Type II in pattern D (also present in aflatoxin-producing GA13-9) results from the deletion of the coding region for amino acid residues 1–181 of NorB and 1–29 of CypA, and the *norB-cypA* intergenic region plus a 32 bp deletion for encoding amino acid residues 300–310 of NorB that are still present in type I. Patterns A and D isolates appear to contain all of the genes in the aflatoxin gene cluster necessary for B-type aflatoxin formation. Pattern B has deletions on the left side of the gene cluster. The three isolates of pattern C have a large deletion in the region between the *cypA* gene and the *avnA* gene. Patterns E–H appear to have a deletion larger than 45 kb. The deletions in patterns E–G isolates extend beyond the left side of the aflatoxin gene cluster. The entire aflatoxin gene cluster in pattern H isolates is deleted, and the right side deletion extends to the *hexA* gene in the downstream sugar utilization gene cluster, *nadA-hexA-glcA-sugR*.

3.2. Sequence breakpoints in pattern C

To determine the sequence breakpoints responsible for pattern C deletion, we made several primers for PCR of regions around the *cypA-aflT* and *verA-avnA* genes. A 0.5 kb PCR product was generated from each genomic DNA of AL3-9, NC5-2, and VA4-4 by primers 5′-agtcgagcagttgcagatga-3′ and 5′-cagggcaatccgaagctcta-3′. The sequences of the three PCR products were compared to those of the aflatoxin gene clusters in *A. flavus* AF13 (AY510451) and *A. parasiticus* NRRL 5862 (AY371490). One breakpoint is in the 3′ untranslated region (UTR) of *cypA* gene. Another breakpoint is located in the coding region of the *verA* gene (Fig. 2). The size of the deleted region is 40 kb.

3.3. Sequence breakpoints in patterns E, G, and H

The deletions on the left side of the aflatoxin gene cluster in the three patterns extend beyond the available

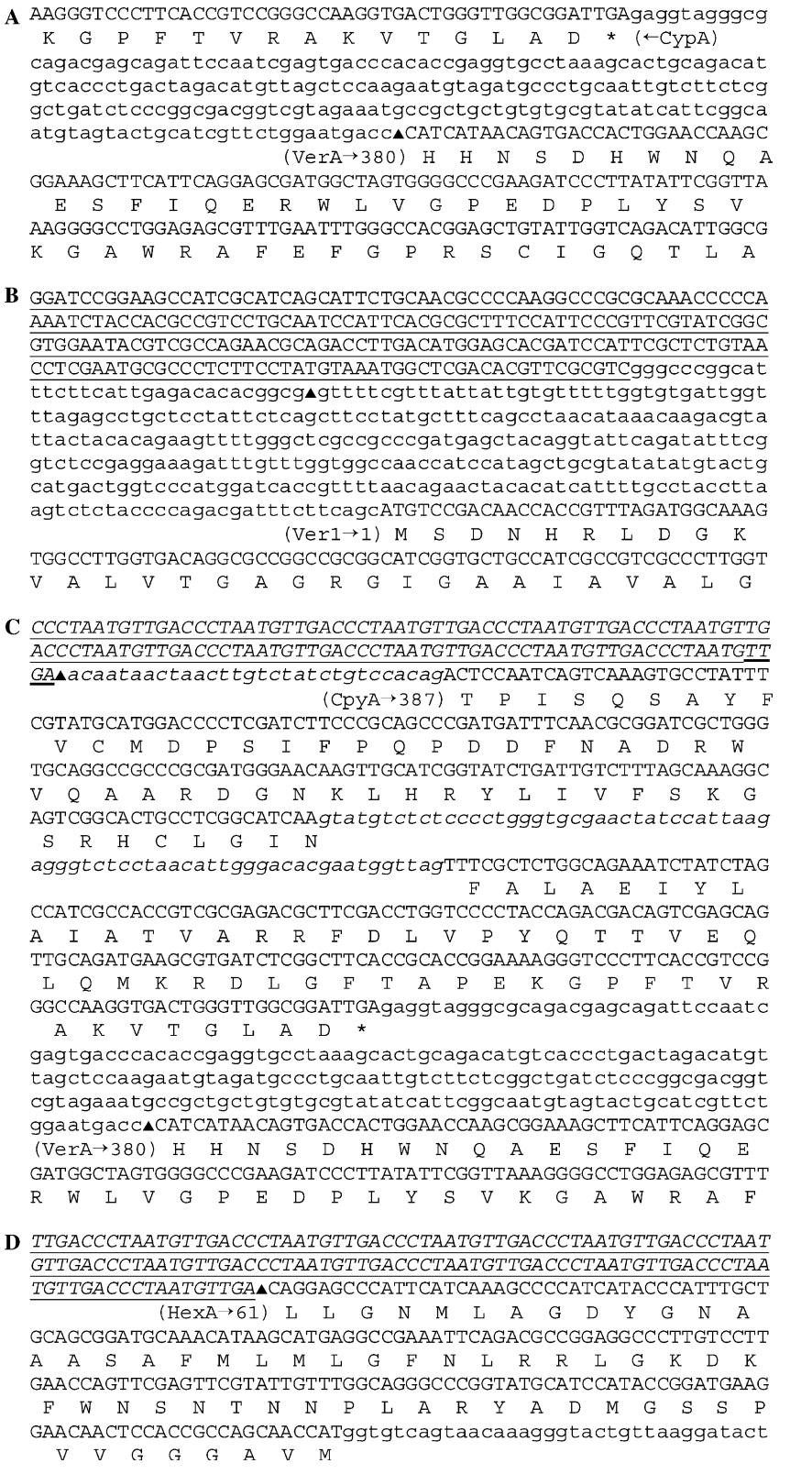

Fig. 2. Sequences around the junctions of breakpoints in the aflatoxin gene cluster and flanking regions. (A) Pattern C deletion. (B) Pattern E deletion. (C) Pattern G deletion. (D) Pattern H deletion. A solid triangle indicates the junction of two breakpoints. The coding sequence is in upper case and the noncoding sequence is in lower case. Putative coding sequence is underlined and in upper case. Intron sequence is italicized and in lower case. The telomeric repeat sequence, CCCTAATGTTGA (= TCAACATTAGGG), is underlined and in italics. An asterisk indicates a stop codon. In pattern G isolates, TX20-9 has a perfect telomeric repeat sequence, CCCTAATGTTGA, next to the junction of the breakpoints, but AL1-4 instead has an imperfect telomeric repeat sequence, CCCTAATGAATA.

sequence. We therefore used an Inverse PCR protocol to determine whether their breakpoints are identical. For pattern E (TX13-5 and TX21-5), only *Bam*HI digested genomic DNA gave a 0.6 kb PCR product. Sequences of both PCR fragments show that a breakpoint is at 300th nt upstream of the start codon of the *ver1* gene (Fig. 2B). The breakpoints at the other end in both isolates are identical, in a gene encoding a protein that has 52% identity to the *A. nidulans AmdA*, a C_2H_2 zinc-finger positive-acting regulatory protein (Lints et al., 1995). Available draft genome sequence of *A. flavus* NRRL 3357 indicates the aflatoxin gene cluster resides about 80 kb from one end of a 2.3 megabasepair (Mb) scaffold. A search of this scaffold did not find any sequence homologous to *amdA*.

For pattern G (AL1-4 and TX20-9), only *Sma*I digested genomic DNA yielded a 0.9 kb PCR product. Sequences of the PCR products revealed that each contained a remaining portion of *cypA* from intron II to 3′ UTR. This results in two junction regions from four sequence breakpoints (Fig. 2C). The breakpoints in the region between the 3′ UTR of *cypA* and *verA* are identical to those found in pattern C isolates, and result in a 40 kb deletion (Figs. 2A and C). Moreover, the left side breakpoint is flanked by 9–10 copies of the perfect repeat sequence, CCCTAATGTTGA. The breakpoints at this end of *cypA* in AL1-4 and TX20-9 are not identical but very close, being only four nucleotides apart (Fig. 2C).

For pattern H (GA1-3, NC7-1, SC5-6, and NRRL 21882), *Bgl*II and *Stu*I treated genomic DNA yielded a 0.8 kb PCR product and a 0.7 kb PCR product, respectively. A comparison of the sequences of the two PCR fragments generated by using the same nested primer indicates they are identical. The breakpoint at the right side of the aflatoxin gene cluster is in the coding region of the *hexA* gene in the sugar utilization gene cluster, at the first nucleotide encoding the amino acid residue #62 of HexA (Fig. 2D). The breakpoint on the left side of the aflatoxin gene cluster is also flanked by 11–12 copies of the repeat sequence, CCCTAATGTTGA, found in pattern G isolates. The breakpoints of the other 14 isolates in pattern H were determined by conventional PCR (see Section 2), and they are identical to those obtained by the Inverse PCR technique.

3.4. Deletion patterns and omtA SNPs in A. flavus isolates belonging to different VCGs

We found that isolates in the same VCG have identical deletion patterns (for example, VCGs 64, 67, 69, and 70 in pattern H), but individual deletion patterns (E, G, and H) contain isolates from different VCGs (Table 1). Since SNPs in the *omtA* gene have been used as a phylogenetic signal (Geiser et al., 2000), we determined the *omtA* SNPs in isolates of patterns B, C, E, F, and G to infer their relationships. Isolates in patterns C and G have identical SNPs in their *omtA* genes (Fig. 3). Isolates in patterns B, E, and F also have identical SNPs. The *omtA* SNPs in patterns A and D, however, are not homogenous (data not shown). On the basis of the *omtA* SNPs, isolates in patterns C and G belong to one subgroup, and isolates in patterns B, E, and F belong to another subgroup. Like deletion patterns, *A. flavus* subgroups contain isolates belonging to different VCGs.

Isolate	Pattern	omtA SNPs
MS1-1	(B)	AAAACAATCCATTCAA
SC3-5	(B)	AAAACAATCCATTCAA
TX13-5	(E)	AAAACAATCCATTCAA
TX21-5	(E)	AAAACAATCCATTCAA
NC7-8	(F)	AAAACAATCCATTCAA
AL3-9	(C)	GCCGGCGGTATCCTTG
NC5-2	(C)	GCCGGCGGTATCCTTG
VA4-4	(C)	GCCGGCGGTATCCTTG
AL1-4	(G)	GCCGGCGGTATCCTTG
TX20-9	(G)	GCCGGCGGTATCCTTG

Fig. 3. Comparison of *omtA* SNPs in different aflatoxin gene cluster deletion patterns. Letters in the parentheses indicate deletion patterns in Fig. 1. Isolates in pattern B have identical *omtA* SNPs, and only two are shown.

4. Discussion

The current study shows that in nonaflatoxigenic *A. flavus* isolates deletion of a part or the entire aflatoxin gene cluster is not rare and the resulting patterns are diverse. Two independent deletions, type I and type II, were found in the *norB-cypA* region (Fig. 1). On the basis of *omtA* SNPs, isolates of pattern E and F, and G likely descend from different *A. flavus* subgroups but contain type I deletion (Fig. 3; Ehrlich et al., 2004). Deletions may preferentially occur in type I isolates, first in the right or left side of the *norB-cypA* region (Fig. 1, patterns B and C) and then toward both directions (Figs. 1, E–H). This scenario is different from the directional deletion proposed for closely related *A. oryzae* isolates (Kusumoto et al., 2000). The *A. oryzae* groups 2 and 3 identified by Kusumoto et al. (2000) have breakpoints estimated to be within 0.8 kb upstream of the *ver1* coding region and within 2.4 kb upstream of the *vbs* coding region, respectively. The breakpoints in *A. flavus* pattern E are probably identical to those in *A. oryzae* group 2. The distance between *vbs* and *avnA* is 15 kb (Fig. 1) in *A. flavus*. Patterns F and G of *A. flavus* thus differ from *A. oryzae* group 3 and represent new types of deletion in the aflatoxin gene cluster. Kusumoto et al. (2003) reported that *A. oryzae* telomeric sequence consists of 9–11 repeats of the dodeca-nucleotide, CCCTAATGTTGA (=TCAACATTAGGG). The same sequence repeats

were found in patterns E and H, which suggests that deletion in each pattern extends to one end of the chromosome. The length of *A. flavus* telomeric repeat is similar to but longer than the telomeric repeat, TTAGGG, found in human, mouse, and several fungal species such as *A. nidulans* (Bhattacharyya and Blackburn, 1997), *Neurospora crassa* (Schechtman, 1990), *Magnaporthe grisea* (Farman and Leong, 1995), and *Fusarium oxysporum* (Garcia-Pedrajas and Roncero, 1996). However, half of the telomeric repeat sequence, TCAACA, is unique to *A. flavus* and the closely related *A. oryzae*.

The draft genome sequence of aflatoxigenic *A. flavus* NRRL 3357 shows that the aflatoxin gene cluster is close to one end of the chromosome. In yeast, the subtelomeric regions are subject to duplications and rearrangements (Liti and Louis, 2005). For example, arsenic resistance genes located in subtelomeric regions in several yeasts undergo rapid evolution involving large-scale genomic rearrangement (Maciaszczyk et al., 2004). *A. nidulans* produces sterigmatocystin, an intermediate in aflatoxin formation, by a biosynthetic gene cluster containing many homologs for aflatoxin production (Brown et al., 1996). The sterigmatocystin gene cluster is located on chromosome IV (Brown et al., 1996) that has a size of 2.9 Mb (Brody and Carbon, 1989). We estimated from ordered supercontigs 9 (1.4 Mb) and 10 (1.4 Mb) that the sterigmatocystin gene cluster is probably located about 100 kb from the chromosomal end (see http://www.broad.mit.edu/annotation/fungi/aspergillus/ for *A. nidulans* genetic maps). Hodges et al. (2000) reported chromosomal translocation occurred within the *stcW* gene (= *A. flavus moxY*) in an echinocandin B-producing strain of *A. nidulans* that does not produce sterigmatocystin. Ehrlich et al. (2005) compared sequences flanking the aflatoxin gene cluster from two *A. flavus* isolates (L and S sclerotial morphotypes), an *A. nomius* isolate, and an unnamed taxon isolate that is phylogenetically intermediate between *A. flavus* and *A. parasiticus*. They showed that, among the three taxa, the left side regions of the aflatoxin gene cluster toward the telomere are highly variable, but the sugar utilization gene cluster on the right side is well conserved. Akita et al. (personal communication) at National Institute of Brewing in Japan have found that only an 18 kb region remains from the left side of the aflatoxin gene cluster to the telomere in *A. oryzae* group 1 and an 8 kb region remains from the *ver1* gene to the telomere in group 2. The chromosomal region from the aflatoxin gene cluster to the telomere thus may be more susceptible to certain selective forces that lead to adaptation or speciation.

The function of aflatoxins is still under debate (Demain and Fang, 2000) and is problematic owing to the observation that aflatoxigenic and nonaflatoxigenic *A. flavus* isolates are equally capable of infecting susceptible crops (Cotty, 1989, 1997). Ehrlich et al. (2005) estimated that aflatoxin production in *A. flavus*, *A. parasiticus*, and *A. nomius* has been maintained for at least 100 million years, which suggests an adaptive value for aflatoxins. Hypotheses for the function of aflatoxins include the removal of excess acetates when fungi grow on carbon-rich sources (Bu'Lock, 1965), the protection of fungi against soil microbial competitors or insect predators (Drummond and Pinnock, 1990; Matsumura and Knight, 1967), and the promotion of conidial or sclerotial development (Calvo et al., 2001; Chang et al., 2001; Cotty, 1989; Wilkinson et al., 2004). Unlike trichothecenes (Proctor et al., 2002), aflatoxins are not acutely phytotoxic and do not act as virulence factors (Hasan, 2001; McLean et al., 1995).

Ward et al. (2002) showed that the virulence-associated trichothecene genes in the *Fusarium graminearum* species complex exhibit considerable genetic variability. They proposed that differences in trichothecene production are maintained by a balancing of selective forces in the chemotype-shaping environment. It is possible that when selective forces necessary to maintain the aflatoxin gene cluster in *A. flavus* either as an efficient horizontal transfer unit (Walton, 2000) or for adaptation to character-shaping niches (Ehrlich et al., 2005) become unimportant, genetic drift leads to further mutations and/or deletions in genes directly or indirectly associated with the production of aflatoxin B_1 and B_2. The loss of production of aflatoxins B_1 and B_2 in many nonaflatoxigenic *A. flavus* isolates is not caused by large deletions or a complete loss of the aflatoxin gene cluster (e.g., patterns A, B, and D; Table 1), but probably results from point mutations (Ehrlich et al., 2004) or small deletions in genes essential for aflatoxin production, such as those having a regulatory role (Calvo et al., 2004) or being involved in the signaling pathway (Hicks et al., 1997). Nonetheless, deletion of the entire aflatoxin gene cluster may be the ultimate consequence in *A. flavus* isolates that no longer produce aflatoxins.

Isolates in the same VCG have identical deletion patterns, but isolates with identical deletion patterns also belong to different VCGs (Table 1). McAlpin et al. (2002) showed that, with few exceptions, *A. flavus* isolates in the same VCG have identical restriction fragment length polymorphism patterns, which indicate they are either clonal or very closely related. In *N. crassa*, heterokaryon (vegetative) incompatibility is mediated by differences in allelic specificity at *het* loci (Glass et al., 2000). At least 11 *het* loci have been identified in *N. crassa* and each *het* locus has two or three allelic specificities (Perkins et al., 2001). At present, we do not sufficiently understand evolutionary processes in *A. flavus* to explain the identical deletion patterns among VCGs. Deletion patterns might have formed before divergence into different VCGs or identical sequence deletions could have occurred independently by a precise mechanism after an *A. flavus* progenitor had diverged into different VCGs. Alternatively, genetic recombination

may be involved as previously proposed (Geiser et al., 1998; Tran-Dinh et al., 1999). The extensive deletions in the aflatoxin gene cluster identified in NRRL 21882 and other candidate nonaflatoxigenic biocontrol *A. flavus* isolates, nonetheless, serve as a safeguard in preventing adverse genetic reversion or recombination, thereby ensuring their safe application to agricultural fields.

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